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14. ABSTRACT

Despite earlier detection and improved therapies, prostate cancer remains a leading cause of cancer-related death among men in the US. Castration resistant prostate cancer (CRPC) represents the most dangerous and presently incurable stage of the disease. In this grant, we explore a new strategy to block the activity of a clinically relevant target known as Hedgehog proteins (Hhs). Hhs comprise a family of extracellular signaling proteins that contribute to the development and progression of prostate cancer. Our strategy to inhibit Hhs as a means to suppress prostate cancer takes aim at a key biosynthetic reaction called cholesterolysis. In cholesterolysis, the oncogenic Hh polypeptide is generated through a peptide cleavage reaction involving cholesterol. Cholesterolysis represents an attractive therapeutic target because: (1) the transformation is unique to Hh proteins, (2) it occurs upstream of all known oncogenic signaling events and (3) it is required for all known Hh signaling events. We are developing and applying tools to discover this new class of compounds. Our approach seeks to combine rational inhibitor design (Major task 1), complementary small molecule screens (Major task 2), and preclinical tests (Major task 3). The patients most likely to benefit from this work are those who are suffering from or at risk of developing CDRP. In this annual report, we summarize work that toward Major task 2 and outline our plans for year 3 to reach a critical milestone – identifying a tight binding, selective inhibitor of Hh cholesterolysis.

15. SUBJECT TERMS

Nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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INTRODUCTION: Hedgehog proteins (Hh), a family of extracellular signaling factors, can promote the malignant expansion of prostate cancer cells into life threatening tumors. Our hypothesis is that chemical inhibitors of Hh protein biosynthesis will suppress this oncogenic activity. We focus on inhibiting a biosynthetic reaction called cholesterolysis, as it is unique to Hh proteins and required for Hh activity (**Fig. 1**). Despite being described more than 20 years ago, no drug-like chemical inhibitor of cholesterolysis is known. In **year 1** of this New Idea Development award, we solved one of the major obstacles in this area by developing a photometric assay to continuously monitor cholesterolysis in multi-well plates. We further showed in year 1 that the assay could be applied to rapidly screen large numbers of molecules in search of chemical inhibitors. In **year 2**, we expand on those results by carrying out the first large-scale high-throughput screening effort for cholesterolysis inhibitors, work that was carried out under contract for services agreement with the Small Molecule Discovery Center at University of California San Francisco. Here, we describe the progress of those efforts. We have completed the contracted tasks set out in the SOW; however the key milestone of discovering a drug-like chemical inhibitor of cholesterolysis has not yet been achieved. As a consequence, we revised our plans for year 3 so as to extend the screening effort in a manner that takes into account the lessons learned from year 2, while keeping the goal of delivering that key milestone of a drug-like chemical inhibitor. Achieving this important and long-sought objective will set the stage for preclinical tests of the compounds anti-cancer activity. In the longer term, the results of our high risk/high reward study provide a springboard toward a new class of tumor targeted, prostate cancer therapeutics.

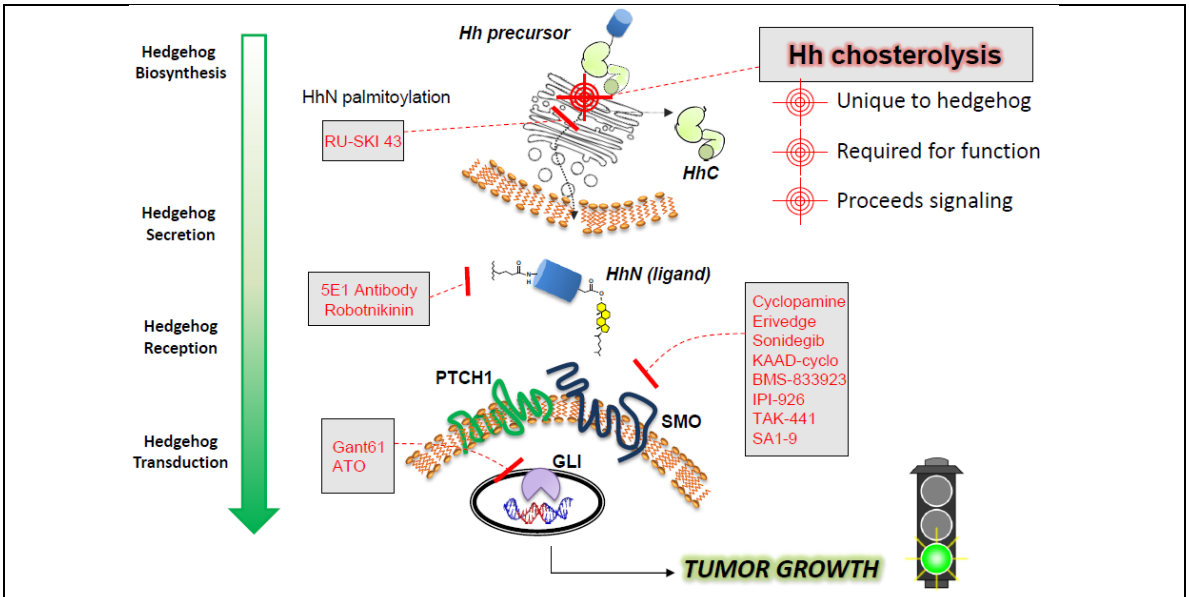

Figure 1. Hedgehog Cholesterolysis – an untapped target to block this oncogenic signaling pathway. Image above depicts oncogenic cell-to-cell communication through a biochemical cascade that begins with hedgehog biosynthesis (top) and culminates in the growth of tumors (bottom). At least two cells are involved, a hedgehog producing cell and a hedgehog sensitive cell. Shaded boxes indicate compounds that have been explored as potential antagonists of the signaling cascade, with the majority of effort focused on blocking a receptor protein called SMO, or smoothened. The overarching goal of this award is to discover first-in-class inhibitors of cholesterolysis.

1. **KEYWORDS:** Prostate, Cancer, Hedgehog, Tumor, Inhibitor, Screening, Therapeutic

2. **ACCOMPLISHMENTS:**
   - **What were the major goals of the project?**
     -

Specific Aim 1	*target dates*	*completion dates or the percentage of completion*
Major Task 1: *To establish arsenic trioxide as the first inhibitor of Hh cholesterolysis.*	Months	
NMR Chemical Shift Mapping to identify changes in global structure of Hh protein upon binding AsIII	1-3	100%
Cysteine Specific, NMR Chemical Shift Mapping, to establish molecular mechanism of action, namely formation of Cys-AsIII-Cys complex	3-5	100%
Structure Activity Analysis to determine the effect of As substituents and valence on the inhibition potency	5-6	100%
Milestone(s) Achieved: Authored manuscript describing the inhibitory activity of AsIII along with structural characterization of binding site	24-26	100% - reprint included in appendix
Specific Aim 2		
Major Task 2: *To identify selective, potent inhibitors of Hh cholesterolysis for application to prostate cancer therapy.*		
High-Throughput Screen of 20,000 drug-like, chemically diverse compounds using an optical assay of Hh cholesterolysis * Screening carried out at the at the Small Molecule Discovery Center, University of	6-24	100%

California San Fransciso, under the direction of Dr. Michelle Arkin		
Tethering Screen of 2,000 drug-like, chemically diverse compounds with purified Hh protein an binding assay * Screening carried out at the at the Small Molecule Discovery Center, University of California San Fransciso, under the direction of Dr. Michelle Arkin	6-24	95%
Milestone(s) Achieved: Identified a panel of ~ 15 active compounds, validated by secondary screening, and possessing IC_{50} values in low μM range.	24-26	October 2016 NOT ACHIEVED
Specific Aim 3		
Major Task 3 *To test lead molecules for effects on release of paracrine Hhs from PCa cell and on paracrine Hh action in PCa xenografts.*		*Reallocated*
Mechanism of action and ADME experiments on two most promising compounds • **Cell lines used:** LNCaP-SHh, LNCaP-AI, LNCaP-C42, LNCaP-LN3, VCaP and PC3	24-30	---.
Local IRB/IACUC Approval	28	---
Preclincal studies in mouse to assess efficacy in shrinking and/or delaying the growth of PCa xenographs • **Mouse xenograft model system used**: LNCaP-AI, PC3	30-36	---
Milestone(s) Achieved: Preclinical validation of Hh cholesterolysis as a druggable target in PCa	36	---

- **What was accomplished under these goals?**

  - *1) Major activities:* Our goal is to discover a novel class of molecules that block biosynthesis of hedgehog proteins, potent cell/cell signaling factors that stimulate PCa progression. In year 2 of this award, we worked collaboratively to meet this goal with the Small Molecule Discovery Center (SMDC) at University of California San Francisco. The SMDC, led by Dr. Michelle Arkin, is expert in drug discovery and has established protocols for conventional small molecule screens and "tethering" screens, the two approaches we chose to pursue to find cholesterolysis inhibitors. Our contribution was to design, validate a cholesterolysis assay, and provide assay components (protein, buffers, detergent, cholesterol) to the SMDC.

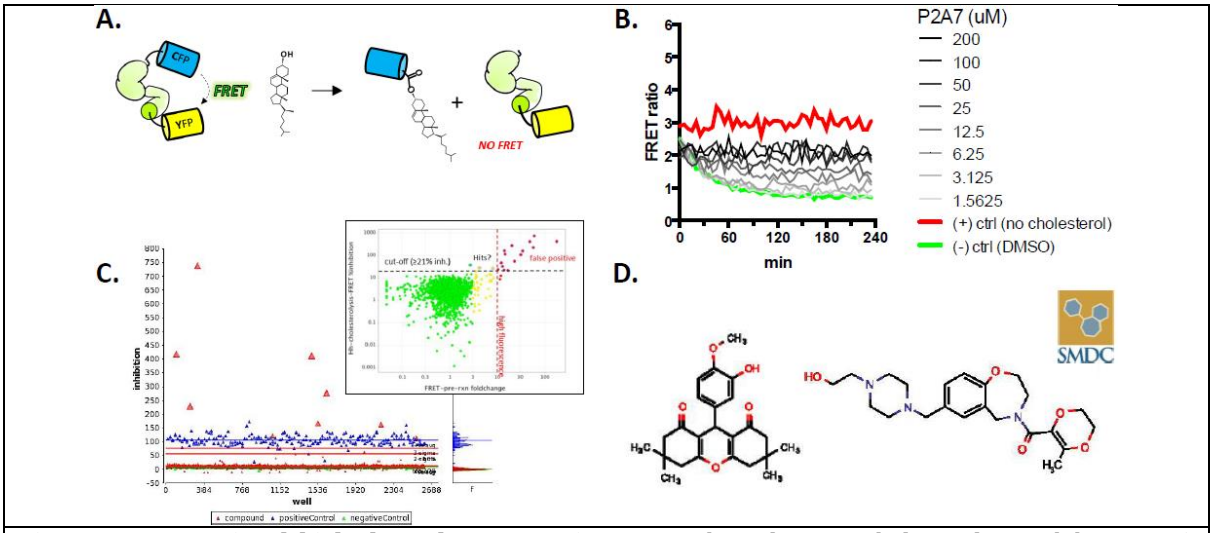

Figure 2. Conventional high throughput screening approach to discover cholesterolysis inhibitors. **A)** Scheme depicting photometric assay of cholesterolysis. This assay, which allows continuous monitoring of cholesterolysis in real time, was developed in year 1 of the award. **B)** Assay validation. Scientists at UCSF Small Molecule Discovery Center carried out cholesterolysis assays in the absence (green trace) and presence (grey to black traces) of increasing concentrations of a small molecule inhibitor of cholesterolysis. The red trace is a negative control. **C)** Assay Results. In this sample, apparent inhibition of cholesterolysis is plotted against ~ 3000 different compounds (well #). The large red triangles indicate potential inhibitors. The inset graph shows how the results were analyzed to eliminate false positives. Typically, false positives were optically active compounds that interfered with the photometric assay. **D)** Example "false positives". These two compounds were discarded from consideration after they failed to inhibit cholesterolysis in follow-up validation assays.

The assay we developed is photometric and uses an engineered **h**edgehog protein (C-H-Y) fused at its N-terminus to **c**yan fluorescent protein and at its C-terminus to **y**ellow fluorescent protein. Using C-H-Y the first two stages of the screening protocols at SMDC, consultation and assay implementation, were completed in year 1 of the grant. In year 2, the SMDC conducted a dry run in which the HTS assay was tested on their instruments to determine feasibility with SMDC robotics and plate readers. The calculated Z' value, a statistical measure of assay quality, was consistently > 0.5, consistent with a high-quality assay. Following that successful dry run, ~ 2000 compounds were tested in a

pilot screen for cholesterolysis inhibitors. All aspects of the screen, including data analysis, were evaluated. Following a successful pilot screen, the HTS campaign began in earnest, working at the fastest pace appropriate for our assay. Although we intended to screen 20,000 compounds, the assay was sufficiently robust to support screening of 80,000 compounds (**Fig. 2**).

Results from the screens were uploaded to the SMDC database called HiTS, which is accessible via password protected web interface. Hits or "Active" wells were those with signals > 3 standard deviations from the mean of the baseline signal. Next, actives were cherry-picked for rescreening using an orthogonal gel-based assay and, by a dose response analysis. We sought compounds that displayed a dose response, with an IC50 <20 μM; however the majority of hits turned out to false positives, molecules that simply interfered with optical measurements rather than inhibited cholesterolysis.

In parallel with the conventional screen, the SMDC conducted a tethering screen using the cholestersolysis-active segment of the hedgehog protein, abbreviated HhC (**Fig 3**). To facilitate the tethering effort, we purified >30 mgs of HhC and sent it to the SMDC. There, HhC was incubated with a library of thiol-containing small-molecules (>4, 000) under conditions that promote disulfide exchange. The intention was to find chemicals that are bound by the active-site cysteine residue of HhC, and thereby interfere with cholesterolysis. Chemicals disulfide-linked to HhC were identified by mass spectrometry. Next, hits from the tethering experiments will be tested as inhibitors of cholesterolysis assay described above. This follow-up work is ongoing at the SMDC. Selected compounds are expected to behave as competitive inhibitors under reducing conditions. Through additional medicinal chemistry, these chemicals could be modified into drug-like inhibitors.

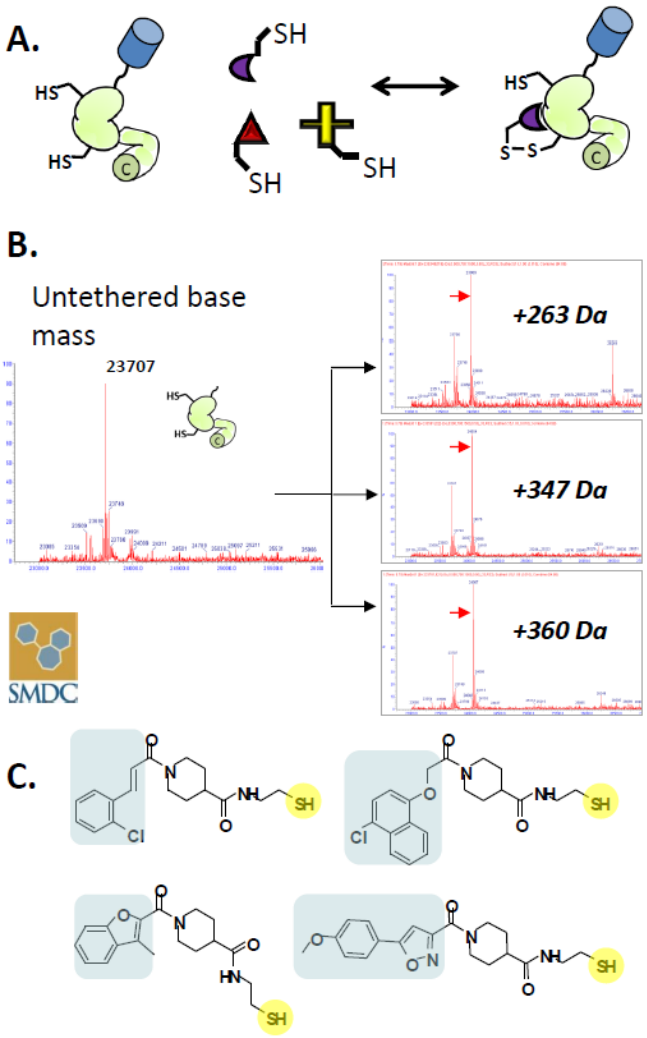

Figure 3. "**Tethering**", a nonconventional approach to the discovery of cholesterolysis inhibitors based on dynamic combinatorial chemistry. **A)** Schematic depiction of a tethering experiment. Here the hedgehog protein containing two cysteine residue are mixed with a collection of thiol-modified small molecules under mildly oxidizing conditions. Over time, the Hh protein and small molecules equilibrate to form mixed disulfides, the compound whose shape is most complementary to the Hh protein will be bound most tightly. **B)** Mass spectrometery analysis of a tethering experiment. To identify small molecules disulfide bonded (tethered) to the Hh protein, the equilibrated solution is analyzed by mass spectrometery. A control reaction containing Hh protein only is analyzed first (left), followed by samples containing Hh protein plus small molecules (right). Increases in the apparent mass of the Hh protein are consistent with stable tethering to a small molecule. Incremental mass increases are compared with the mass of the small molecules in the tethering experiment to identify the binding compound. **C)** Selection of potential cholesterolysis inhibitors that were discovered by tethering with Hh protein. These compounds are being tested in follow up assays by the team at UCSF.

- *2) specific objectives;* As stated in the SOW, our objective was to facilitate through collaboration the first high throughput screening campaign to find drug-like chemical inhibitors of hedgehog cholesterolysis. The approach was two-pronged: (1) we would carry out a conventional screen using a large collection of chemically diverse small molecules (2) we would carry out a non-conventional screen using a smaller library of small molecules, each one bearing a thiol functional group (-SH) for the purpose of engaging one of the active-site cysteine residues of hedgehog protein in a disulfide bond.
- *3) Significant results or key outcomes, including major findings, developments, or conclusions (both **positive** and **negative**).* The major

positive outcome of year 2, was that the experimental system we developed for screening very large numbers of chemicals in search of cholesterolysis inhibitors was successful in practice. In fact, ours is the first high throughput screen against this protein target. The SMDC researchers were impressed by the ease of screening, the low cost of the assay components, as well as the assays reproducibility. The major **negative** outcome is that our goal of discovering a cholesterolysis inhibitor was not met in year 2. From our discussions with SMDC, the general belief is that the Hh is a "difficult target", with an unconventional binding site for small molecules (i.e. chemical inhibitors). Compounding the problem, we have no structural information about that unconventional binding site on the Hh protein, because the protein has proved recalcitrant to x-ray crystallography and NMR, the two workhorse techniques of structural biology. Our plans for year 3 to achieve the key milestone of discovering a cholesterolysis inhibitor are outlined below.

- *4) Other achievements.* Although we were not successful in year 2 in our quest to find a drug-like inhibitor of cholesterolysis, we were productive in two related projects, which led to peer-reviewed publications (see Appendix). First, we continued to probe the chemical mechanism of Hh cholesterolysis through mutagenesis coupled with structural studies, culminating in a publication in high-impact *Journal of the American Chemical Society*. This type of fundamental study brings further refinement to our understanding of the protein we are targeting, and hence supports the overall project goal. Second, we discovered an unexpected interaction between hedgehog proteins and the prostate-cancer drugs, abiraterone (Zytiga) and galeterone. The off-target reaction generates covalent hedgehog–drug conjugates. Cell-based reporter assays indicate that these conjugate activate hedgehog signaling when present in the low nanomolar range. Because hedgehog signaling is implicated in prostate cancer progression, and abiraterone is administered to treat advanced stages of the disease, this off-target interaction may have therapeutic significance. This work was published last month in the journal, *ChemMedChem*.

- *5) Stated goals not met.* Our goal of discovering drug-like chemical inhibitor of cholesterolysis was not met in year 2.

- *6) Discussion of stated goals not met.* The reason(s) our goal was not met of discovering a drug-like compound that could be advanced to preclinical

studies is not clear at this time. In terms of the scope of work, the experiments were completed on time using the assay system the PI developed, and in fact, we tested 60,000 more compounds than we had originally budgeted for in the contract with SMDC. Despite the effort, the high risk/high reward process of drug discovery did not yield. As a result, the goal must be pursued into year 3, and our original plans for year 3, postponed. Our revised approach for year 3 involves replacing *quantity* of compounds tested with *quality* of compounds tested (**Fig 4**). Specifically, we plan to test smaller chemical libraries (2000-4000 members) that are enriched in compounds that are either (1) electrophilic or (2) sterol-like. These two compound classes resemble the inhibitors discovered in year 1, where we were successful in identifying chemical inhibitors, albeit not drug-like inhibitors.

- **What opportunities for training and professional development has the project provided?**
  - *Nothing to report*
- **How were the results disseminated to communities of interest?**
  - *This past summer, the PI presented results from this project at the PCRP IMPACT meeting in Towson Maryland.*
- **What do you plan to do during the next reporting period to accomplish the goals?**

In year 3 of the award, our plan had been to test drug-like compounds, identified in year 2, for anti-cancer activity against prostate cancer cells in vitro and in mice. Thus, year 3 activity for the award is contingent on success in year 2. The cell and animal work originally planned for year 3 were to take place at Vancouver Prostate Centre under the guidance of Dr. Ralph Buttyan. Our milestone of finding a drug-like compound has not been reached. Nonetheless, both I and Dr. Buttyan remain committed to delivering a drug-like compound that targets hedgehog and thereby validates the New Idea.

Because no selective inhibitors of hedgehog cholesterolysis were found in year 2, we requested that funds be re-allocated for year 3 of the award (**Fig 4**). The funds will be used to screen smaller, more focused libraries for inhibitors. The **first** chemical library will be purchased from Asinex Inc, which contains molecules with mild electrophilic moieties that could potentially interact with cysteine residues in the target hedgehog protein. The diversity of scaffolds in the Asinex library, ranges from Natural Product-like systems to Macrocycles, and creates multiple opportunities for drug discovery. Our attention was drawn to the Asinex library based on our findings in year 1 of the award that the hedgehog protein can be inhibited by electrophilic compounds. The **second** chemical library to be screened is the NHRCore™ Library from Chembridge, containing a computationally selected library of more than 3,000 leadlike small molecules. NHRCore compounds are generated

from published compounds with activity against nuclear hormone receptor targets, including androgen receptor. We were drawn to this library because hedgehog cholesterolysis can be inhibited be steroid-like molecules, such as epicoprostanol.

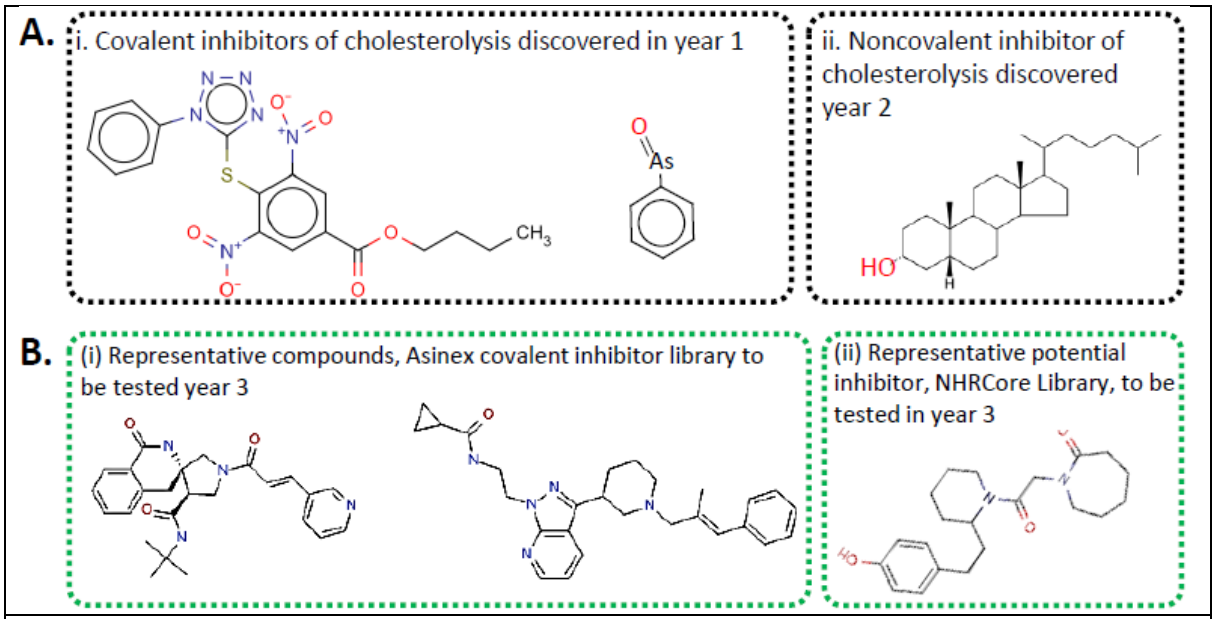

FIGURE 4. Cholesterolysis inhibitors discovered to date through this project provide a rational for further discovery work in **year 3**. **A)** Chemical inhibitors of cholesterolysis discovered in year 1 of the award that block hedgehog by covalent (i) and noncovalent (ii) mechanisms. The chemicals provide proof of concept but do not possess the physico-chemical characteristics of drug-like molecules. **B)** Compounds to be screened in year 3 include electrophiles, expected to inactivate hedgehog covalently (i), and steroid analogues, expected to inactivate hedgehog non-covalently. These molecules have drug-like characteristics

Thus our plan for year 3 is to extend the chemical screening, although through a more directed search, with the aim of identifying drug-like chemical inhibitors that are suitable for preclinical trials of anti-prostate cancer activity.

IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**
  - *Nothing to report*
- **What was the impact on other disciplines?**
  - *Nothing to Report*
- **What was the impact on technology transfer?**
  - *Nothing to Report*
- **What was the impact on society beyond science and technology?**
  - *Nothing to Report.*

3. **CHANGES/PROBLEMS:**
    - **Changes in approach and reasons for change**
        - As described above, we our extending the screening work into year 3 with the aim of discovering a drug-like cholesterolysis inhibitor. This change of plans has been communicated to the Scientific Officer and the budget office.
    - **Actual or anticipated problems or delays and actions or plans to resolve them**
        - *Nothing to report*
    - **Changes that had a significant impact on expenditures**
        - *Nothing to Report*
    - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
        - *Nothing to Report.*
    - **Significant changes in use or care of human subjects**
        - *Nothing to Report*
    - **Significant changes in use or care of vertebrate animals.**
        - *Nothing to Report*
    - **Significant changes in use of biohazards and/or select agents**
        - *Nothing to Report*

4. **PRODUCTS:**
    - **Publications, conference papers, and presentations**
        - **(1)** A Single Aspartate Coordinates Two Catalytic Steps in Hedgehog Autoprocessing. Xie J, Owen T, Xia K, Callahan B, Wang C. *J Am Chem Soc.* 2016 Aug 31;138(34):10806-9.
        - Status of publication (**published**).
        - Acknowledgement of federal support (**yes**/no).
        - **(2)** Hedgehog Proteins Consume Steroidal CYP17A1 Antagonists: Potential Therapeutic Significance in Advanced Prostate Cancer. Bordeau BM, Ciulla DA, Callahan BP. *ChemMedChem.* 2016 Sep 20;11(18):1983-6.
        - Status of publication (**published**);
        - Acknowledgement of federal support (**yes**/no).

    - **Conference papers and presentations.**:
    - (1) Brian P. Callahan , Timothy Owen , Georget Ngoje, Brandon Bordeau , Chunyu Wang , Jian Xie Targeting hedgehog protein biosynthesis with small molecules; a search for inhibitors and unanticipated discovery of anti-androgen activators. *IMPACT, PCRP 2016 Towson, MA*

- **Website(s) or other Internet site(s)**
  (1) Department website of PI (Callahan), announcing federal support: https://www.binghamton.edu/chemistry/news/
- (2) Callahan research group page, with links to publicationshttp://www.binghamton.edu/chemistry/people/callahan/callahan.html s

5. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	*Brian Callahan, PhD*
Project Role:	*Principal Investigator*
Nearest person month worked:	*9*
Contribution to Project:	*Dr. Callahan designed experiments, analyzed results, and wrote manuscripts related to the project.*

Name:	*Timothy Owen*
Project Role:	*Graduate Student*
Nearest person month worked:	*5*
Contribution to Project:	*Mr. Owen carried out experiments, analyzed results, and assisted in writing manuscripts related to the project.*

Name:	*Dan Ciulla*
Project Role:	*Lab Technician*
Nearest person month worked:	*5*
Contribution to Project:	*Mr. Ciulla carried out experiments, analyzed results, and assisted in writing manuscripts related to the project.*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - *Nothing to report*

- **What other organizations were involved as partners?**
  - *Provide the following information for each partnership:*
    - **Organization Name:** *University of California San Francisco*
    - **Location of Organization:** *San Francisco, California*
    - **Partner's contribution to the projec**t *(identify one or more)*
      - **Facilities** *High Throughput Chemical Screening*
      - **Collaboration** *Prof. Michelle Arkin*
      - **Personnel exchanges** *Tim Owen (Binghamton University) travelled to Dr. Arkin's lab to assist with the high throughput screening efforts*
    -
    - **Organization Name:** *Rensselaer Polytechnic Institute*
    - **Location of Organization:** *Troy, New York*
    - **Partner's contribution to the projec**t *(identify one or more)*
      - **Facilities** *NMR facility for biomolecular structural studies related to project*
      - **Collaboration** *Prof. Chunyu Wang (Biology Department) collaborated with project staff*
      - **Personnel exchanges** *(e.g., project staff and/or partner's staff use each other's facilities, work at each other's site)*; and
      - **Other.**

6. **SPECIAL REPORTING REQUIREMENTS**
   - **Not Applicable**

7. **APPENDICES:**

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Communication

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A Single Aspartate Coordinates Two Catalytic Steps in Hedgehog Autoprocessing

Jian Xie,[†] Timothy Owen,[‡] Ke Xia,[§,⊥] Brian Callahan,[‡] and Chunyu Wang*[,†,§,∥,⊥]

[†]Biochemistry and Biophysics Graduate Program, [§]Department of Chemistry and Chemical Biology, [∥]Department of Biological Sciences, and [⊥]Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 Eighth Street, Troy, New York 12180, United States

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Supporting Information

ABSTRACT: Hedgehog (Hh) signaling is driven by the cholesterol-modified Hh ligand, generated by autoprocessing of Hh precursor protein. Two steps in Hh autoprocessing, N−S acyl shift and transesterification, must be coupled for efficient Hh cholesteroylation and downstream signal transduction. In the present study, we show that a conserved aspartate residue, D46 of the Hh autoprocessing domain, coordinates these two catalytic steps. Mutagenesis demonstrated that D46 suppresses non-native Hh precursor autoprocessing and is indispensable for transesterification with cholesterol. NMR measurements indicated that D46 has a pK_a of 5.6, ∼2 units above the expected pK_a of aspartate, due to a hydrogen-bond between protonated D46 and a catalytic cysteine residue. However, the deprotonated form of D46 side chain is also essential, because a D46N mutation cannot mediate cholesteroylation. On the basis of these data, we propose that the proton shuttling of D46 side chain mechanistically couples the two steps of Hh cholesteroylation.

Post-translational modification by cholesterol activates Hedgehog (Hh) family proteins,[1] which are secreted signaling ligands with crucial roles in development and cancer.[2,3] Cholesteroylation of Hh slows the rate of extracellular diffusion, giving rise to finely regulated signaling gradients during embryogenesis.[4] Mutagenesis studies show that abolishing cholesteroylation prevents Hh ligand secretion, resulting in the degradation of premature Hh ligand, and blockade of downstream signaling events.[5−8] In humans, defects in Hh cholesteroylation are associated with holoproencephaly (HPE), a congenital syndrome that impairs brain development.[9−11] Cholesteroylation of Hh is carried out by the autoprocessing activity of Hh precursor[12,13] (Figure 1A).

The 45 kDa Hh precursor is composed of two domains, an N-terminal signaling domain (HhN) and a C-terminal autoprocessing domain (HhC) (Figure 1A and 1B). HhC has two functional segments, a Hint (Hedgehog/intein) module and a sterol-recognition region (SRR) (Figure 1B). During Hh autoprocessing, HhN is cleaved from the precursor and covalently linked to cholesterol at the C-terminus. This transformation is catalyzed by HhC in two steps (Figure 1A):

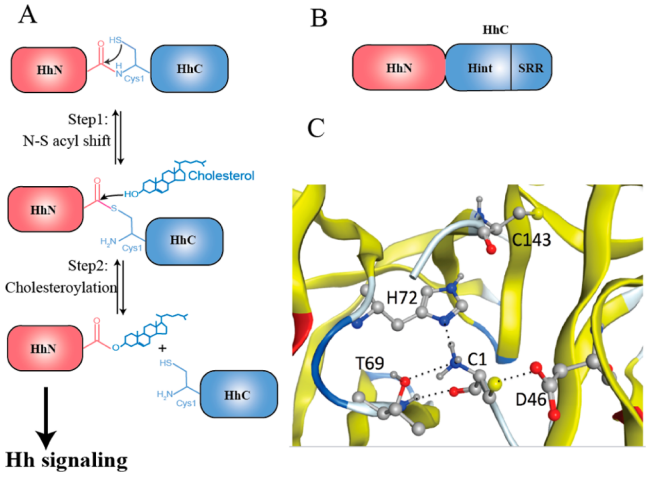

Figure 1. Catalytic steps, domain structure, and active site in Hedgehog autoprocessing. (A) Two steps in Hh autoprocessing mechanism. Hh = Hedgehog; HhN = N-terminal domain of Hh; HhC = C-terminal domain of Hh. (B) Domain organization of the Hh precursor protein. SRR = sterol recognition region which binds cholesterol. (C) Active site of the Hint domain, composed of C1, D46, T69, H72, and C143. Dashed lines denote hydrogen bonds.

1. N−S acyl shift—the conserved Cys 1 of HhC carries out a nucleophilic attack on the carbonyl of the last glycine residue of HhN (G-1), resulting in a thioester intermediate. An identical step occurs in intein-mediated protein splicing, also catalyzed by the Hint module.[13]
2. Transesterification—the hydroxyl group of a cholesterol molecule bound to SRR attacks the thioester, displacing and cholesteroylating HhN.

These two steps of Hh autoprocessing are well coordinated. When the Hh autoprocessing reaction products were analyzed by MS, only cholesteroylated HhN was observed.[3,13] If the two steps are not closely coupled, the thioester intermediate can react with nucleophiles other than cholesterol, precluding production of the lipidated HhN ligand required for proper Hh signaling.

Hh autoprocessing is dependent on the autocatalytic activity of the Hint domain (Figure 1B). The crystal structure of

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Drosophila melanogaster (Dme) Hint domain[13] shows an active site composed of conserved polar residues: C1, D46, T69, H72, and C143 (Figure 1C). C1 is the nucleophile attacking the scissile bond carbonyl (step 1), supported by loss of activity in C1A mutants.[14] T69 and H72 form the signature "TXXH" motif important for N−S acyl shift in both Hh and inteins. D46 and C143 do not appear to have a mechanistic counterpart in inteins but are known to be crucial for autoprocessing.[13,15] C143 can form an internal disulfide with C1, proposed to be important for Hh folding.[13] However, the mechanistic role of D46 is poorly understood.

D46 Inhibits Unproductive Autoprocessing of Hh Precursor. We used a recently developed FRET-based assay for Hh autoprocessing[16] to probe the role of D46. The key construct, C-H-Y, has cyan (C) and yellow (Y) fluorescent proteins[17] fused to the N- and C-termini of the *Dme* HhC domain. In the Hh precursor state, the CFP and YFP are close to each other and the fusion protein exhibits FRET; this FRET is lost when the fluorescent proteins are separated from each other through normal autoprocessing in the presence of cholesterol (Figure 2A). C-H-Y also reacts with non-native

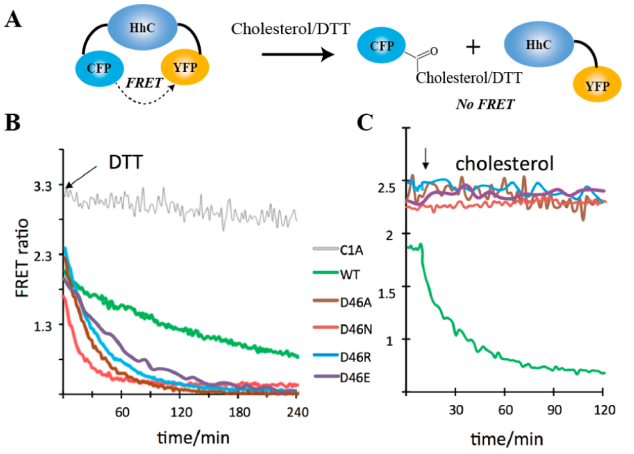

Figure 2. FRET-based DTT-cleavage and autoprocessing assays demonstrate D46 inhibits premature Hh precursor cleavage and is indispensable for cholesteroylation. (A) Schematics of the FRET assays. (B) D46 WT has much slower DTT-mediated N-terminal cleavage than D46A, D46N, D46E, and D46R mutants. (C) Cholesteroylation is abolished in all four D46 mutants.

nucleophiles, such as dithiothreitol (DTT), which can substitute for cholesterol in attacking the thioester intermediate. We compared the wild-type to four variants: the alanine point mutant, D46A; an isosteric mutant, D46N; a charge reversed mutant, D46R; and D46E, which changes the side chain by only a methylene group. All four expressed as soluble proteins in *E. coli* and exhibited strong FRET signals consistent with proper folding.

We first assayed the reactivity of C-H-Y and the D46 point mutants toward DTT as a means of assessing the first step of autoprocessing, N−S acyl shift (Figure 2B). In the presence of DTT (0.2 M), the D46 WT reacted at a rate of (8.4 ± 0.2) × 10^{-5} s^{-1}, indicating the presence of an internal thioester. As a negative control for the assay, we used a C1A mutant, where a hydrogen atom replaces the native thiol group. This mutant was insensitive to added DTT, as apparent from its stable FRET signal. Interestingly, all four D46 mutations *accelerated* the non-native reaction toward DTT compared with WT (Figure 2B), with rates of (54 ± 4) × 10^{-5} s^{-1} for D46A, (83 ± 5) × 10^{-5} s^{-1} for D46N, (55 ± 3) × 10^{-5} s^{-1} for D46N, and (26 ± 1) × 10^{-5} s^{-1} for D46E. Gel-based cleavage assay showed similar results (Figure S4). Thus, the native D46 has a role in restricting non-native autoprocessing, most likely by manipulating the N−S acyl shift equilibrium or by blocking access to the thioester intermediate. This autoinhibitory effect on promiscuous cleavage is fitting for a coordination residue, which should control the first step before the second step is ready to proceed.

D46 Is Crucial for the Transesterification Step. Using the FRET assay, we next probed the effect of D46 mutations on second step of Hh autoprocessing. The WT protein exhibited a rapid decline in the FRET ratio following addition of cholesterol (0.25 mM), in accord with our earlier study.[16] By contrast, there was *negligible* change in FRET for D46N, D46E, D46R and D46A, indicating that these mutations abolish cholesteroylation activity (Figure 2C). The lack of activity with cholesterol is not a consequence of defective N−S acyl shift (first step), because the robust cleavage mediated by DTT indicates that thioester formation is intact (Figure 2B). Therefore, D46 not only restricts off pathway activity, but is essential for transesterification to cholesterol, suggesting that D46 likely mediates crucial interactions with the substrate. Indeed, extending the native side chain in D46E by a methylene group blocks activity toward cholesterol. The D46N mutant has a side chain very similar to the WT except for the negative charge of aspartate. The fact that D46N can not mediate cholesteroylation suggests that the deprotonated aspartate side chain is important for transesterification. We suspected that the acid−base equilibrium of D46 is crucial for Hh autoprocessing, leading us to examine the pK_a of D46 with solution NMR.

pK_a of D46 Is Elevated. A strikingly high pK_a value of 5.6 was obtained by NMR titration in the precursor (Figure 3A and Figure S1), composed of 5 HhN residues and Hint domain (Table S1). A similar pK_a of 5.8 was observed in the Hint domain (Figure S3), indicating that the presence of HhN residues do not affect D46 pK_a. Both values are about two units above) the normal pK_a of aspartate side chain. High pK_a of active-site aspartate usually points to the mechanistic importance of the protonated aspartic side chain.

pK_a Values of D46 and C1 Are Coupled. Next we examined the structural basis of the elevated pK_a of D46. Because D46 and C1 are close in the 3D structure (Figure 1C), we examined how C1A mutation affects the pK_a of D46. Interestingly, the C1A mutation decreases the D46 pK_a to 4.2 (Figure 3B), close to the regular pK_a value of aspartate, demonstrating that D46 pK_a is enhanced by the absolutely conserved C1 side chain. We then tested whether D46 in turn influences the side chain of C1. We monitored cysteine ^{13}CB chemical shift in ^{1}H−^{13}C HSQC using a specifically cysteine ^{13}CB labeled sample (Figure S2).[18] In the WT precursor, C1 has a pK_a of 5.1 (Figure 3C), more than 3 units lower than the normal pK_a of cysteine, ~8.3. The D46A mutation increased C1 pK_a to 6.3 (Figure 3D). Therefore, the elevated pK_a of D46 is coupled to the decreased pK_a of C1.

The pK_a coupling suggests a direct interaction between D46 and C1, and can be explained by a hydrogen bond between C1 thiolate and D46 carboxyl (Figure 3E). As a general rule, a positive charge lowers the pK_a of nearby ionizable groups, whereas a nearby negative charge raises the pK_a. In Figure 3E, the negative charge of the C1 thiolate increases D46 pK_a, while the positive partial charge of the carboxyl hydrogen decreases the C1 pK_a. Because D46 inhibits spurious Hh precursor

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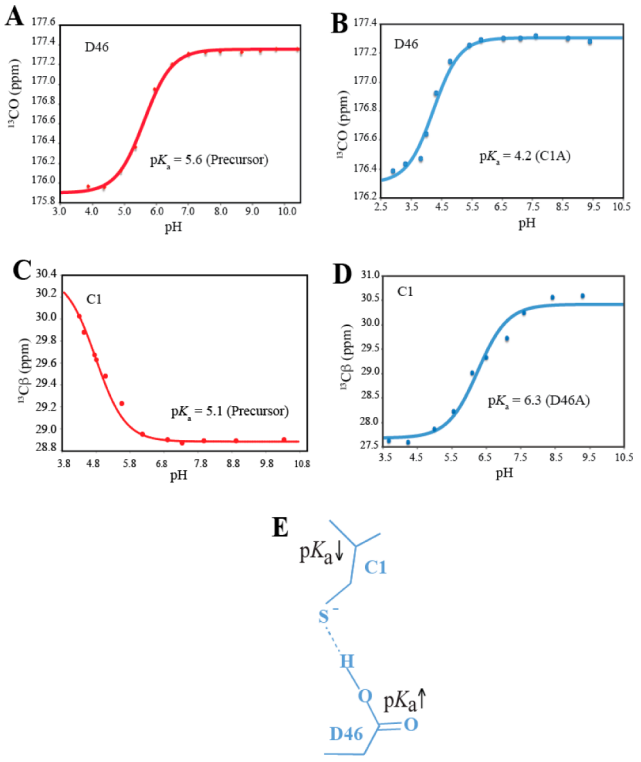

Figure 3. pK_a coupling between D46 and C1. (A) D46 pK_a is elevated to 5.6, determined by ^{13}CO chemical shift titration with HB(CB)CO (see Figure S1). (B) C1A mutation lowers D46 pK_a to normal. (C) C1 has a depressed pK_a of 5.1 and a pK_a of 6.3 with a D46A mutation (D), based on ^{13}CB chemical shift changes with pH. (E) The structural basis for pK_a shift and coupling between C1 and D46.

reactivity (Figure 2B), D46 likely holds C1 thiolate in a nonproductive conformation or alternatively stabilizes the thioester, before cholesteroylation can proceed, serving the role of a coordination residue. Similar pK_a coupling has been observed between a conserved aspartate (but not homologous to D46) and C1 in an intein.[19] pK_a matching has also been observed for catalytic interactions in other enzymes.[20]

D46 Coordinates the Two Steps through Side-Chain Proton Shuttling. On the basis of the above data, we propose a novel mechanism for D46 coordination:

1. D46 carboxyl stabilizes the C1 thiolate but holds it in an inactive conformation, pointing away from scissile bond carbonyl (Figure 4). D46 should therefore inhibit N−S acyl shift mediated Hh cleavage by DTT. Indeed, as demonstrated in Figure 2B, D46 has a much slower rate of DTT cleavage than four D46 mutants. D46 should also have an elevated pK_a, due to the nearby negative charge of C1 thiolate, confirmed by NMR titration (Figure 3A,B).
2. We hypothesize that when cholesterol binds to Hh, the hydroxyl of cholesterol interacts with D46, lowering its pK_a due to the partially positive charge of the hydroxyl proton. D46 deprotonates, liberating C1 thiolate to carry out the N−S acyl shift. D46 may donate its proton to a nearby water molecule, H72, or the incipient amine group of C1.
3. In a coupled step, the newly deprotonated D46 side chain is poised to serve as a general base to activate the hydroxyl group of bound cholesterol for attack at the thioester.

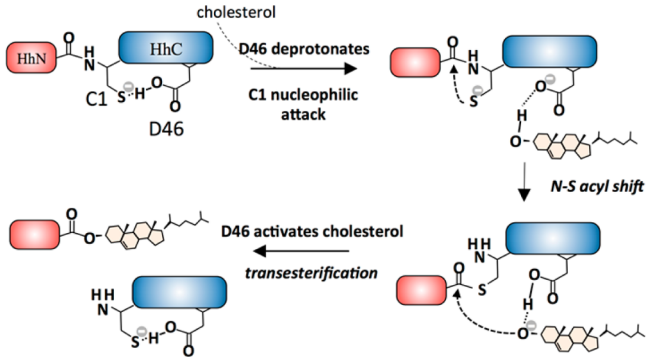

Figure 4. Coordination mechanism of D46 in the two catalytic steps of Hh autoprocessing, in which the protonation and deprotonation of D46 plays a pivotal role.

This scheme links N−S acyl shift and transesterification through proton shuttling by D46, and suppresses premature N−S acyl shift and thioester cleavage, ensuring the fidelity of Hh autoprocessing. Further, the mechanism explains the unique conservation of this aspartate side chain in Hh Intein domains/modules.

Many enzymes catalyze multistep reactions. Without proper coordination in these enzymes, side reactions will occur and prevent the formation of the intended product. The coordination mechanism of D46 in Hh autoprocessing provides an example of how conformational change of a side chain coupled with proton shuttling can drive the precise progression of complicated steps at an active site. Even in nonenzymatic systems, such as the chlorine transporters (CLC), similar behavior is observed. In CLC, an active site glutamate cycles through ionization states at three sites.[21] At two sites, the negatively charged glutamate side chain replaces bound chlorines; at a third site, it gains a proton, which neatly accounts for the exchange stoichiometry of 2 Cl^- for 1 H^+ in these transporters.

Hh signaling, driven by the Hh ligand, plays vital roles in both embryogenesis and cancer. Hh autoprocessing, which generates the Hh ligand, thus occupies a unique position at the very origin of the Hh signaling cascade. Abnormal Hh autoprocessing in development leads to congenital diseases. Recently, we provided evidence that abnormal Hh autoprocessing may be linked to zinc deficiency[22] and metabolism of antiprostate cancer drugs.[23] Our results in this paper improve our understanding of Hh's unique autoprocessing mechanism while providing a foothold[18] to correct aberrant Hh levels in developmental disorders and cancer.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06928.

Additional methods and data, including Figures S1−S4 and Table S1 (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Porter, J. A.; Young, K. E.; Beachy, P. A. *Science* **1996**, *274* (5285), 255.
(2) Briscoe, J.; Thérond, P. P. *Nat. Rev. Mol. Cell Biol.* **2013**, *14* (7), 418.
(3) Xie, J.; Owen, T.; Xia, K.; Singh, A. V.; Tou, E.; Li, L.; Arduini, B.; Li, H.; Wan, L. Q.; Callahan, B.; Wang, C. *J. Biol. Chem.* **2015**, *290* (18), 11591.
(4) Porter, J. A.; von Kessler, D. P.; Ekker, S. C.; Young, K. E.; Lee, J. J.; Moses, K.; Beachy, P. A. *Nature* **1995**, *374* (6520), 363.
(5) Lee, J. J.; Ekker, S. C.; von Kessler, D. P.; Porter, J. a; Sun, B. I.; Beachy, P. a. *Science* **1994**, *266* (5190), 1528.
(6) Guy, R. K. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97* (13), 7307.
(7) Chen, X.; Tukachinsky, H.; Huang, C.-H.; Jao, C.; Chu, Y.-R.; Tang, H.-Y.; Mueller, B.; Schulman, S.; Rapoport, T. a; Salic, A. *J. Cell Biol.* **2011**, *192* (5), 825.
(8) Tukachinsky, H.; Kuzmickas, R. P.; Jao, C. Y.; Liu, J.; Salic, A. *Cell Rep.* **2012**, *2* (2), 308.
(9) Belloni, E.; Muenke, M.; Roessler, E.; Traverse, G.; Siegel-Bartelt, J.; Frumkin, A.; Mitchell, H. F.; Donis-Keller, H.; Helms, C.; Hing, A. V.; Heng, H. H.; Koop, B.; Martindale, D.; Rommens, J. M.; Tsui, L. C.; Scherer, S. W. *Nat. Genet.* **1996**, *14* (3), 353.
(10) Roessler, E.; Belloni, E.; Gaudenz, K.; Vargas, F.; Scherer, S. W.; Tsui, L. C.; Muenke, M. *Hum. Mol. Genet.* **1997**, *6* (11), 1847.
(11) Roessler, E.; El-Jaick, K. B.; Dubourg, C.; Vélez, J. I.; Solomon, B. D.; Pineda-Alvarez, D. E.; Lacbawan, F.; Zhou, N.; Ouspenskaia, M.; Paulussen, A.; Smeets, H. J.; Hehr, U.; Bendavid, C.; Bale, S.; Odent, S.; David, V.; Muenke, M. *Hum. Mutat.* **2009**, *30* (10), E921.
(12) Porter, J. A.; Ekker, S. C.; Park, W.-J.; von Kessler, D. P.; Young, K. E.; Chen, C.-H.; Ma, Y.; Woods, A. S.; Cotter, R. J.; Koonin, E. V.; Beachy, P. A. *Cell* **1996**, *86* (1), 21.
(13) Hall, T. M.; Porter, J. a; Young, K. E.; Koonin, E. V.; Beachy, P. a; Leahy, D. J. *Cell* **1997**, *91* (1), 85.
(14) Hall, T. M.; Porter, J. A.; Beachy, P. A.; Leahy, D. J. *Nature* **1995**, *378* (6553), 212.
(15) Chen, X.; Tukachinsky, H.; Huang, C.-H.; Jao, C.; Chu, Y.-R.; Tang, H.-Y.; Mueller, B.; Schulman, S.; Rapoport, T. A.; Salic, A. *J. Cell Biol.* **2011**, *192* (5), 825.
(16) Owen, T. S.; Ngoje, G.; Lageman, T. J.; Bordeau, B. M.; Belfort, M.; Callahan, B. P. *Anal. Biochem.* 2015, *488* (1−5).
(17) Nguyen, A. W.; Daugherty, P. S. *Nat. Biotechnol.* **2005**, *23* (3), 355.
(18) Owen, T. S.; Xie, X. J.; Laraway, B.; Ngoje, G.; Wang, C.; Callahan, B. P. *ChemBioChem* **2015**, *16* (1), 55.
(19) Du, Z.; Zheng, Y.; Patterson, M.; Liu, Y.; Wang, C. *J. Am. Chem. Soc.* **2011**, *133*, 10275.
(20) Zhao, Q.; Abeygunawardana, C.; Talalay, P.; Mildvan, a S. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93* (16), 8220.
(21) Feng, L.; Campbell, E. B.; MacKinnon, R.; Feng, L.; MacKinnon, R.; Campbell, E. B.; MacKinnon, R. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (29), 11699.
(22) Xie, J.; Owen, T.; Xia, K.; Singh, A. V.; Tou, E.; Li, L.; Arduini, B.; Li, H.; Wan, L. Q.; Callahan, B.; Wang, C. *J. Biol. Chem.* **2015**, *290* (18), 11591.
(23) Bordeau, B. M.; Ciulla, D. A.; Callahan, B. P. *ChemMedChem* **2016**, DOI: 10.1002/cmdc.201600238.

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CHEMMEDCHEM Communications

Hedgehog Proteins Consume Steroidal CYP17A1 Antagonists: Potential Therapeutic Significance in Advanced Prostate Cancer

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Abiraterone, a potent inhibitor of the human enzyme CYP17A1 (cytochrome P450c17), provides a last line of defense against ectopic androgenesis in advanced prostate cancer. Herein we report an unprecedented off-target interaction between abiraterone and oncogenic hedgehog proteins. Our experiments indicate that abiraterone and its structural congener, galeterone, can replace cholesterol as a substrate in a specialized biosynthetic event of hedgehog proteins, known as cholesterolysis. The off-target reaction generates covalent hedgehog–drug conjugates. Cell-based reporter assays indicate that these conjugates activate hedgehog signaling when present in the low nanomolar range. Because hedgehog signaling is implicated in prostate cancer progression, and abiraterone is administered to treat advanced stages of the disease, this off-target interaction may have therapeutic significance.

Hedgehog (Hh) proteins serve as cell-signaling ligands involved in embryo development, whereas deregulated signaling by Hh is implicated in cancer.[1] Multiple studies link aberrant signaling by hedgehog to prostate cancer progression.[2] One attractive target to modulate the activity of Hh is the protein's unique biosynthesis.[3] Hh proteins are expressed in the form of a self-catalytic, multidomain precursor protein. The Hh signaling ligand, HhN, is released from this precursor by peptide bond cholesterolysis, a cleavage/lipidation event unique to the Hh family[4] (Figure 1 A). Cholesterolysis occurs in the secretory pathway,[5] before signaling, and represents one of two Hh-specific lipidations.[6] The reaction is brought about by the precursor's C-terminal segment, HhC. Mutations in HhC that deactivate cholesterolysis result in endoplasmic reticulum (ER)-associated degradation of the precursor, effectively shutting off downstream signaling.[7]

During small-molecule screens intended to find inhibitors of Hh cholesterolysis, we noticed an unexpected activity *enhancement* in the presence of abiraterone, a powerful antagonist of the steroidogenic enzyme, CYP17A1. Abiraterone (**A**) is an active metabolite of abiraterone acetate (Zytiga™), currently prescribed for the treatment of advanced prostate cancer.[8]

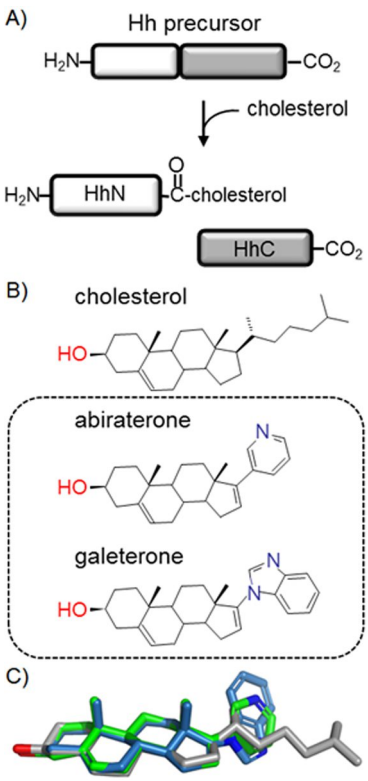

Figure 1. Hedgehog protein biogenesis. A) Cholesterolysis: Hh precursors react autonomously with substrate cholesterol, generating cholesterol-modified signaling ligand, HhN, and the cholesterolysis domain, HhC. B) Comparison of the native substrate with steroidal anti-androgens, abiraterone and galeterone, depicted in simple bond line format. C) Structure overlay of cholesterol (grey), abiraterone (green), galeterone (blue).

Similar activation of Hh cholesterolysis was observed with galeterone (**G**), a structural analogue of abiraterone and clinical candidate for prostate cancer therapy. Results herein indicate that both steroidal agents are accepted by the Hh precursor as alternative substrates for cholesterolysis. The reaction generates covalent HhN–drug conjugates (HhN–**A** and HhN–**G**) in place of native, Hh–cholesterol (HhN–**chol**). The potential adverse effects of this off-target interaction are compounded by our observation that HhN–**A** and HhN–**G** activate Hh signaling in the low nanomolar range, similar to HhN–**chol**. Along with identifying a new, potentially oncogenic activity of Hh in drug metabolism, these findings expand the polypharmacological profile of two clinically significant anticancer agents.

We were drawn to abiraterone and galeterone on the basis of clinical significance and molecular structure. As mentioned,

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Supporting information (preparation and characterization of proteins, cell signaling assays and analysis, mass spectrometry, and kinetic methods) and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/cmdc.201600238.

A is in clinical use for treating castration-resistant prostate cancer, a generally incurable stage of the disease; **G** is under clinical study for the same condition.[9] By inhibiting CYP17A1, these compounds block ectopic androgen biosynthesis, post-castration.[10] Androgen signaling is a long-recognized driver of prostate cancer,[11] and ~70% of patients respond to **A**, with an average life extension of 4 to 6 months.[12] The mechanisms of chemoresistance to anti-androgens remain a subject of active debate.[13] As can be seen in Figure 1B, both **A** and **G** possess a steroidal ring system with a pyridyl or benzimidazole moiety appended to the C17 atom, replacing the native isooctyl "tail" of cholesterol. Given their structural similarity (Figure 1C), we asked whether these compounds might compete with cholesterol for binding by HhC.

We used an activity assay to evaluate interactions of **A** and **G** with the cholesterolysis-active HhC segment from *Drosophila melanogaster*. The assay continuously monitors activity by changes in the fluorescence of FRET-active proteins attached to HhC.[3c] Cyan fluorescent protein, serving as the FRET donor, is fused to the N terminus of HhC, replacing the signaling ligand; yellow fluorescent protein, the FRET acceptor, is fused to C terminus of HhC (Figure 2A). The construct, C–H–Y, exhibits FRET that decays at a saturable rate with added cholesterol, owing to donor–acceptor separation.[3b,c]

In preliminary experiments, **A** was tested as an inhibitor of C–H–Y cholesterolysis. Prior to initiating the reaction with cholesterol, we monitored the FRET ratio of C–H–Y in the presence of added **A** for a period of 20 min; we found in previous work that subtle changes to the FRET ratio during pre-incubation can serve as a marker of Hh compound interaction.[3b] Unlike those earlier observations, however, we noticed that the addition of **A** induced a dramatic change in the FRET ratio of C–H–Y solutions, eventually reaching a baseline value (Figure 2B). This behavior, also observed during pre-incubation with **G**, suggested that C–H–Y was cleaving to form products, C–sterol and H–Y. Analysis of the reaction mixture by SDS-PAGE indeed showed a loss of precursor protein and accumulation of products (Supporting Information Figure S1). In subsequent experiments, we found that C–H–Y reacted with **A** in a concentration-dependent manner; a similar result was obtained with **G** (Figure 2C). The apparent affinity (K_M value) and maximum rates of reaction with the compounds are in the neighborhood of substrate cholesterol (Supporting Information Table S1). Remarkably, the substrate activity of galeterone appears to exceed that of cholesterol in our assay. Thus, **A** and **G** appear to compete with cholesterol, not as inhibitors but as alternative substrates.

We validated the substrate activity of **A** and **G** through secondary assays involving a chimeric Hh precursor, where the human sonic hedgehog ligand (20 kDa) is fused to the cholesterolysis-active HhC of *D. melanogaster*, more closely mimicking a native Hh precursor. We devised this chimeric precursor, SHhN–DHhC, following difficulties we and others encountered expressing recombinant full-length human (and *Drosophila*) Hh precursor.[3b] Activity of SHhN–DHhC toward cholesterol has been established by SDS-PAGE analysis and mass spectrometry.[3b,14] Reactions of SHhN–DHhC in solutions containing **A**, **G**, or cholesterol monitored by SDS-PAGE are shown in Figure 2D. Consistent with the kinetic studies above, results indicate that both synthetic sterols stimulate processing of the precursor into SHhN–sterol and DHhC. Moreover, results of substrate competition experiments, in which cholesterol and **A** (or **G**)

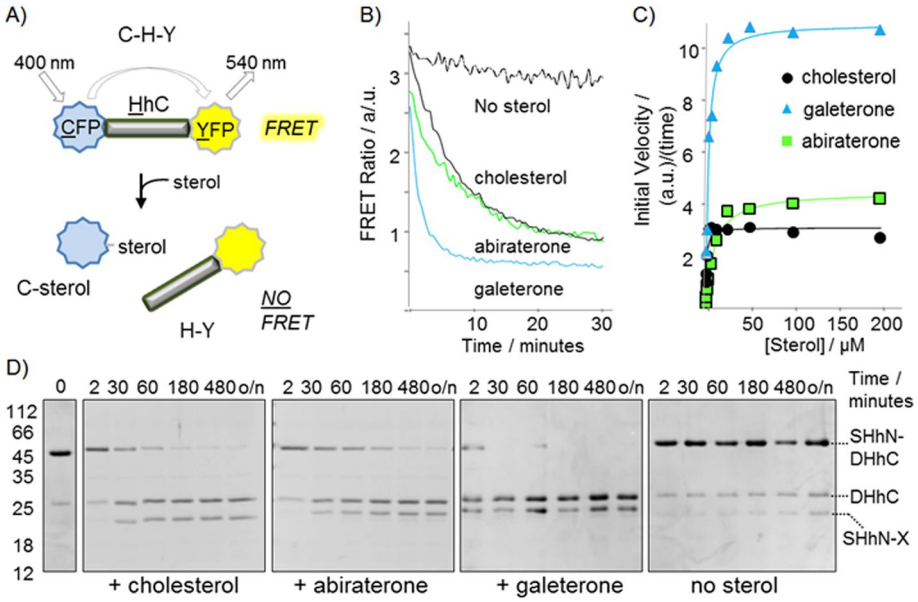

Figure 2. Abiraterone and galeterone can replace cholesterol in hedgehog protein cholesterolysis. A) Optical reporter, C–H–Y, to monitor Hh activity. B) Kinetic traces showing signal from C–H–Y in buffered solution ± sterols (25 μM). C) Michaelis–Menten plot of C–H–Y initial velocity plotted as function of increasing concentration of cholesterol, abiraterone, and galeterone. Solid lines represent the expected kinetic behavior using the following K_M values: cholesterol, 1×10^{-6} M; abiraterone, 12×10^{-6} M; galeterone, 3×10^{-6} M. D) Abiraterone and galeterone are active as substrates with chimeric hedgehog precursor, SHhN–DHhC. SDS-PAGE based assay showing time-dependent processing of SHhN–DHhC precursor processing in the presence of the indicated sterols (250 μM), or buffer alone at 22 °C. M_r: SHhN–DHhC, 46 kDa; SHhN, 20 kDa; DHhC, 26 kDa.

were added together to solutions of SHhN–DHhC, display product partitioning in ratios expected by the kinetic analysis (Supporting Information Figure S2). Thus, **A** is slightly less active as an alternative substrate compared with cholesterol, while **G** appears to surpass the substrate activity of cholesterol. Inspection of the gel also shows that the resulting SHhN conjugates exhibit varying mobility depending on the presence and identity of the attached sterol. Aberrant migration of SHhN is also consistent with covalent modification by a sterol molecule.[15] To establish conjugation of **A** and **G** to the SHhN C terminus, molecular masses of the trypsin-digested proteins were determined. Mass increases of the C-terminal peptide of 332.2 Da for SHhN–**A**, and 371.25 Da for SHhN–**G** were apparent, in accord with esterification of **A** and **G**, respectively, to the terminal glycine of SHhN (Supporting Information Figure S3).

Finally, we assessed the potential impact on downstream Hh signaling if **A** or **G** were to replace native cholesterol during Hh biosynthesis in the cell. It is known that cholesterol modification enhances but is not required for Hh signaling in vitro.[16] On the other hand, the potential influence of the appended sterol's structure on Hh signaling has not yet been evaluated. By in vitro steroylation of the chimeric precursor, we generated SHhN–**A**, SHhN–**G** along with SHhN–**chol** and sterol-free SHhN in amounts suitable for signaling assays with Hh-responsive C3H10T1/2 cells[17] (Figure 3 A). Two isoleucine residues at the N terminus of SHhN provided a surrogate for the protein's native fatty acid modification.[18] As a negative control, we prepared cholesterol-modified human desert HhN ligand (DHhN–**chol**), which exhibits ~100-fold weaker signaling than SHhN.[19] The purity of the conjugates was determined by SDS-PAGE (Supporting Information Figure S4) and by RP-HPLC (Figure 3 B).

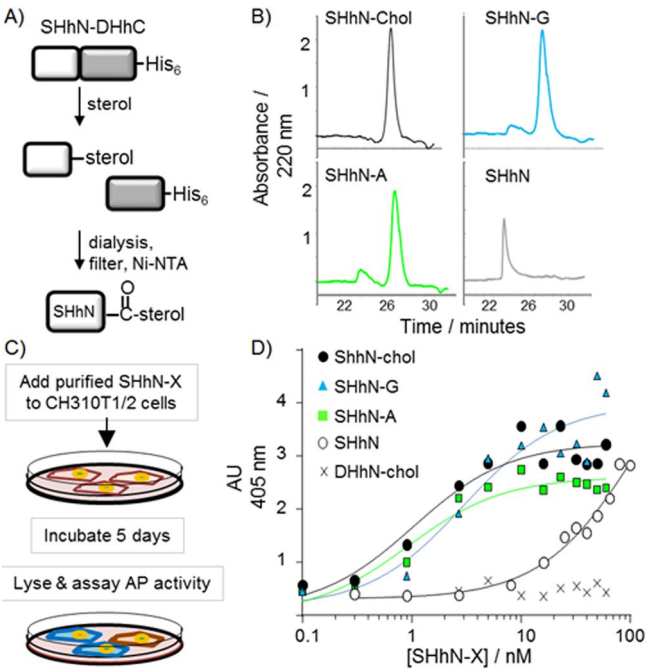

Figure 3. Hh–drug conjugates activate the hedgehog pathway. A) Schematic for the preparation of Hh–ligand conjugates using in vitro steroylation. B) RP-HPLC elution profiles of sterol-free and sterol-modified SHhN. Proteins were separated over a C_4 column using an acetonitrile gradient; longer retention times indicate increased hydrophobicity. C) Schematic of Hh signaling assay using endogenous alkaline phosphatase as reporter. D) Sensitivity of Hh signaling pathway to Hh–drug conjugates. The plot shows averaged alkaline phosphatase activity from CH310T1/2 cells plotted as a function of increasing concentrations of the indicated Hh ligand ($n > 6$, over three trials). Dose–response curves show expected behavior using the following EC_{50} values: SHhN–**chol**, 1×10^{-9} M; SHhN-**A**, 1×10^{-9} M; SHhN-**G** 3×10^{-9} M; sterol-free SHhN; 100×10^{-9} M.

SHhN–**A** and SHhN–**G** mimic the signaling potency of SHhN–**chol**. Activation of the Hh pathway in C3H10T1/2 cells promotes differentiation into osteoblasts, with an ensuing increase in alkaline phosphatase activity[20] (Figure 3 C). In Figure 3 D, AP activity in C3H10T1/2 cells is plotted as a function of increasing concentration of SHhN–X. In accord with earlier studies, SHhN–**chol** activates the pathway when present at single-digit nanomolar concentrations, whereas cholesterol-free Hh is less potent by a factor of > 10.[16a] Our negative control, DHhN–**chol**, did not activate Hh signaling over the range we tested, consistent with earlier work.[19] When the native lipid of SHhN is replaced by **A** or **G**, pathway activation remained robust, with EC_{50} values in the low nanomolar range. We obtained a rank order in terms of potency of SHhN–**chol** ≈ SHhN–**A** > SHhN–**G**. An alternative staining assay with C3H10T1/2 cells produced similar results. Thus, a degree of functional promiscuity exists toward the sterol of SHhN both in ligand biosynthesis and in signal transduction.

Binding to more than a single protein target can sometimes enhance a drug's efficacy;[21] however, the polypharmacology of **A** and **G** identified here seem to point in the opposite direction. Our studies suggest that interactions with Hh could divert **A** and **G** from the intended therapeutic target, CYP17A1, and generate unnatural Hh conjugates competent to activate a tumorigenic pathway. From the perspective of treatment, identifying an off-target interaction could prove useful to guide the design of next-generation analogues that: a) retain CYP17A1 inhibition, and b) bypass covalent interaction with Hh. A 3-keto analogue of abiraterone,[22] Δ^4-abiraterone, along with nonsteroidal anti-androgens, provide logical points of departure.[23] The present findings also support a new oncogenic role of Hh in drug metabolism, a consequence of sterol promiscuity in Hh precursor cholesterolysis.[4] Hh's self-lipidation activity is thereby brought into sharper focus as an important target for prostate cancer. Selective inhibitors hold promise of suppressing Hh biosynthesis while rescuing tumor sensitivity to a currently approved anti-androgen.

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[1] a) A. Hanna, L. A. Shevde, *Mol. Cancer* **2016**, *15*, 24; b) J. Briscoe, P. P. Therond, *Nat. Rev. Mol. Cell Biol.* **2013**, *14*, 416 – 429.

[2] a) A. Shaw, J. Gipp, W. Bushman, *Oncogene* **2009**, *28*, 4480 – 4490; b) S. S. Karhadkar, G. S. Bova, N. Abdallah, S. Dhara, D. Gardner, A. Maitra, J. T. Isaacs, D. M. Berman, P. A. Beachy, *Nature* **2004**, *431*, 707 – 712; c) P. Sanchez, A. M. Hernandez, B. Stecca, A. J. Kahler, A. M. DeGueme, A. Barrett, M. Beyna, M. W. Datta, S. Datta, A. Ruiz i Altaba, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 12561 – 12566; d) M. Chen, M. A. Feuerstein, E. Levina, P. S. Baghel, R. D. Carkner, M. J. Tanner, M. Shtutman, F. Vacherot, S. Terry, A. de La Taille, R. Buttyan, *Mol. Cancer* **2010**, *9*, 89; e) Y. C. Peng, C. M. Levine, S. Zahid, E. L. Wilson, A. L. Joyner, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 20611 – 20616; f) H. Li, W. Liu, W. Chen, J. Zhu, C. X. Deng, G. P. Rodgers, *Sci. Rep.* **2015**, *5*, 16974; g) Y. C. Peng, A. L. Joyner, *Dev. Biol.* **2015**, *400*, 94 – 104.

[3] a) S. Q. Jiang, H. Paulus, *J. Biomol. Screening* **2010**, *15*, 1082 – 1087; b) T. S. Owen, X. J. Xie, B. Laraway, G. Ngoje, C. Wang, B. P. Callahan, *ChemBioChem* **2015**, *16*, 55 – 58; c) T. S. Owen, G. Ngoje, T. J. Lageman, B. M. Bordeau, M. Belfort, B. P. Callahan, *Anal. Biochem.* **2015**, *488*, 1 – 5; d) P. Ciepla, A. I. Magee, E. W. Tate, *Biochem. Soc. Trans.* **2015**, *43*, 262 – 267.

[4] R. K. Mann, P. A. Beachy, *Annu. Rev. Biochem.* **2004**, *73*, 891 – 923.

[5] X. Chen, H. Tukachinsky, C. H. Huang, C. Jao, Y. R. Chu, H. Y. Tang, B. Mueller, S. Schulman, T. A. Rapoport, A. Salic, *J. Cell Biol.* **2011**, *192*, 825 – 838.

[6] a) J. A. Buglino, M. D. Resh, *J. Biol. Chem.* **2008**, *283*, 22076 – 22088; b) J. Long, R. Tokhunts, W. M. Old, S. Houel, J. Rodgriguez-Blanco, S. Singh, N. Schilling, A. J. Capobianco, N. G. Ahn, D. J. Robbins, *Cell Rep.* **2015**, *10*, 1280 – 1287.

[7] a) E. Roessler, E. Belloni, K. Gaudenz, F. Vargas, S. W. Scherer, L. C. Tsui, M. Muenke, *Hum. Mol. Genet.* **1997**, *6*, 1847 – 1853; b) R. K. Guy, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 7307 – 7312.

[8] L. N. Yin, Q. Z. Hu, *Nat. Rev. Urol.* **2014**, *11*, 32 – 42.

[9] A. Bryce, C. J. Ryan, *Clin. Pharmacol. Ther.* **2012**, *91*, 101 – 108.

[10] a) G. Attard, A. S. Belldegrun, J. S. de Bono, *BJU Int.* **2005**, *96*, 1241 – 1246; b) S. T. Page, D. W. Lin, E. A. Mostaghel, D. L. Hess, L. D. True, J. K. Amory, P. S. Nelson, A. M. Matsumoto, W. J. Bremner, *J. Clin. Endocrinol. Metab.* **2006**, *91*, 3850 – 3856; c) R. B. Montgomery, E. A. Mostaghel, R. Vessella, D. L. Hess, T. F. Kalhorn, C. S. Higano, L. D. True, P. S. Nelson, *Cancer Res.* **2008**, *68*, 4447 – 4454; d) C. Cai, S. Chen, P. Ng, G. J. Bubley, P. S. Nelson, E. A. Mostaghel, B. Marck, A. M. Matsumoto, N. I. Simon, H. Wang, S. P. Balk, *Cancer Res.* **2011**, *71*, 6503 – 6513.

[11] C. Huggins, R. E. Stevens, Jr., C. V. Hodges, *Arch. Surg.* **1941**, *43*, 209 – 223.

[12] J. S. de Bono, C. J. Logothetis, A. Molina, K. Fizazi, S. North, L. Chu, K. N. Chi, R. J. Jones, O. B. Goodman, Jr., F. Saad, J. N. Staffurth, P. Mainwaring, S. Harland, T. W. Flaig, T. E. Hutson, T. Cheng, H. Patterson, J. D. Hainsworth, C. J. Ryan, C. N. Sternberg, S. L. Ellard, A. Flechon, M. Saleh, M. Scholz, E. Efstathiou, A. Zivi, D. Bianchini, Y. Loriot, N. Chieffo, T. Kheoh, C. M. Haqq, H. I. Scher, *N. Engl. J. Med.* **2011**, *364*, 1995 – 2005.

[13] P. A. Watson, V. K. Arora, C. L. Sawyers, *Nat. Rev. Cancer* **2015**, *15*, 701 – 711.

[14] J. Xie, T. Owen, K. Xia, A. V. Singh, E. Tou, L. Li, B. Arduini, H. Li, L. Q. Wan, B. Callahan, C. Wang, *J. Biol. Chem.* **2015**, *290*, 11591 – 11600.

[15] J. A. Porter, K. E. Young, P. A. Beachy, *Science* **1996**, *274*, 255 – 259.

[16] a) D. P. Baker, F. R. Taylor, R. B. Pepinsky, *Methods Mol. Biol.* **2007**, *397*, 1 – 22; b) V. K. Grover, J. G. Valadez, A. B. Bowman, M. K. Cooper, *PLOS ONE* **2011**, *6*, e21353.

[17] R. B. Pepinsky, C. Zeng, D. Wen, P. Rayhorn, D. P. Baker, K. P. Williams, S. A. Bixler, C. M. Ambrose, E. A. Garber, K. Miatkowski, F. R. Taylor, E. A. Wang, A. Galdes, *J. Biol. Chem.* **1998**, *273*, 14037 – 14045.

[18] F. R. Taylor, D. Wen, E. A. Garber, A. N. Carmillo, D. P. Baker, R. M. Arduini, K. P. Williams, P. H. Weinreb, P. Rayhorn, X. Hronowski, A. Whitty, E. S. Day, A. Boriack-Sjodin, R. I. Shapiro, A. Galdes, R. B. Pepinsky, *Biochemistry* **2001**, *40*, 4359 – 4371.

[19] S. Pathi, S. Pagan-Westphal, D. P. Baker, E. A. Garber, P. Rayhorn, D. Bumcrot, C. J. Tabin, R. Blake Pepinsky, K. P. Williams, *Mech. Dev.* **2001**, *106*, 107 – 117.

[20] T. Nakamura, T. Aikawa, M. Iwamoto-Enomoto, M. Iwamoto, Y. Higuchi, M. Pacifici, N. Kinto, A. Yamaguchi, S. Noji, K. Kurisu, T. Matsuya, *Biochem. Biophys. Res. Commun.* **1997**, *237*, 465 – 469.

[21] M. L. Bolognesi, A. Cavalli, *ChemMedChem* **2016**, *11*, 1190 – 1192.

[22] Z. Li, A. C. Bishop, M. Alyamani, J. A. Garcia, R. Dreicer, D. Bunch, J. Liu, S. K. Upadhyay, R. J. Auchus, N. Sharifi, *Nature* **2015**, *523*, 347 – 351.

[23] C. Tran, S. Ouk, N. J. Clegg, Y. Chen, P. A. Watson, V. Arora, J. Wongvipat, P. M. Smith-Jones, D. Yoo, A. Kwon, T. Wasielewska, D. Welsbie, C. D. Chen, C. S. Higano, T. M. Beer, D. T. Hung, H. I. Scher, M. E. Jung, C. L. Sawyers, *Science* **2009**, *324*, 787 – 790.

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